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ASSESSMENT AND CHARACTERIZATION OF *COLLETOTRICHUM* SPECIES CAUSING BITTER ROT DISEASE OF APPLE IN QUETTA

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ABSTRACT

Colletotrichum species pose a significant threat to the economy of Pakistan, primarily due to the favorable environmental conditions that facilitate pathogen dispersal. This research was conducted to accurately identify *Colletotrichum* species and study their pathogenic behavior, which is responsible for pre-harvest bitter rot disease in apple fruits in Quetta, Pakistan. A survey was conducted across ten locations in Quetta to assess disease prevalence and collect samples. The findings revealed a disease incidence of 39.22% in one season and 32.56% in another. The symptoms included sunken brown lesions, measuring 1-4 cm in diameter, on the fruit surface, containing small, pinhead-sized black fruiting structures. A total of 130 fungal isolates were obtained from infected samples and were primarily categorized into three groups (A, B, C) based on distinct cultural and morphological characteristics. Pathogenicity test confirmed *Colletotrichum* as the causative pathogen. Further, ten isolates from each group were subjected to molecular analysis to identify the *Colletotrichum* species using internal transcribed spacer (ITS), beta-tubulin (TUB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene regions. Sequence analysis identified the fungal isolates as three *Colletotrichum* species: Group (A) *C. acutatum*, Group (B) *C. gloeosporioides*, and Group (C) *C. siamense*. To our knowledge, this is the first comprehensive study of *Colletotrichum* spp. causing bitter rot disease of apples in Pakistan. The findings of our research will pave the way for future disease management strategies, aiming to prevent this disease from becoming a more serious threat in the region.

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INTRODUCTION

Apple (*Malus domestica* Borkh.) is a member of the *Rosaceae* family, cultivated primarily in the cold, hilly regions of the world (Powell, 1985). It is the third-most produced fruit globally after Banana and Watermelon (FAO, 2023; Shahbandeh, 2023). Pakistan produces 604,000 million tons of apple from an area of 78,300 hectares, which enables it to secure the 23rd position in global production and the 9th in area under apple cultivation. Balochistan accounts for 94.1% and 93.8% of the country's total apple production and area, respectively. In fact, Quetta is one of the most suitable districts for apple cultivation (GOP, 2023).

Balochistan is known as "the fruit basket of the country," playing a pivotal role in the national fruit production (Barrech *et al.*, 2023). However, the region faces significant challenges from various plant diseases, particularly those caused by fungal pathogens.

The per-unit area production (9 tons ha^{-1}) is much less than major apple-growing regions in the world,

including Italy (43.72 tons ha^{-1}) and the United States (35.61 tons ha^{-1}) (Tareen *et al.*, 2020). The apple orchards in Quetta possess the capacity for a more abundant harvest, yet several diseases are impeding this potential. Apple is susceptible to a wide array of diseases that have a significant impact on both fruit quantity and quality, including apple scab, powdery mildew, *Alternaria* leaf blotch, frogeye leaf spot, and bitter rot. The bitter rot disease (BRD) caused by *Colletotrichum* is a destructive both before and after harvest and is responsible for huge economic losses around the world, especially in places where it rains a lot and is warm (Peres *et al.*, 2005; Munir *et al.*, 2016). Depending upon the susceptibility of the cultivar, environmental condition, and destructive nature of the pathogen, BRD causes 10–100% damage to apple fruit in different regions across the globe, including China, Spain, and Italy (Chen *et al.*, 2022; Cabrefiga *et al.*, 2022; Wenneker *et al.*, 2021).

Worldwide, tropical and temperate regions widely distribute the genus *Colletotrichum*, which comprises over 185 species (Jayawardena *et al.*, 2016; Weir *et al.*, 2012). These *Colletotrichum* species are responsible for the anthracnose and fruit rot diseases of a wide array of host plants, including almond, apple, bell pepper, peach, strawberry (De Silva *et al.*, 2021; Khodadadi *et al.*, 2020; Tariq *et al.*, 2017; Tan *et al.*, 2022; Caro *et al.*, 2023).

The favorable environmental conditions in Balochistan, including temperature and humidity levels, facilitate the dispersal and proliferation of these pathogens, exacerbating the impact on apple orchards as described by Martin and Peter (2023) and Salotti *et al.* (2022).

The symptoms of bitter rot disease appear as small grayish brown spots that turn brownish circular lesions on the surface of apple fruits, often accompanied by fruiting bodies (acervuli) arranged in concentric rings (Ben Struble and Keitt, 1950; Sutton *et al.*, 2014; Beever *et al.*, 1995).

Despite the economic importance of apple cultivation in Quetta, there has been limited research focused on understanding the specific *Colletotrichum* species responsible for bitter rot and their pathogenic behavior.

Considering the importance of *Collettotrichum*, species the study was planned to conduct survey and assess the prevalence and incidence of BRD affecting apple trees in Quetta; analyse the morphological & molecular characterization; identify the *Colletotrichum* species causing BRD; confirmation of pathogenic behaviour of obtained isolates; investigate phylogeny relationship with already submitted sequences around the globe.

By identifying the specific *Colletotrichum* species and understanding their pathogenic mechanisms, this research seeks to contribute valuable insights for the development of effective disease management strategies. The findings are expected to pave the way for future research and interventions aimed at mitigating the impact of bitter rot disease on apple production in the region, thereby supporting the sustainability of Balochistan's fruit industry.

MATERIALS AND METHODS

Description of Study Area

Apple growing is unique in the semi-arid region. The study was conducted in 10 apple growing areas in district Quetta, Balochistan. It has semi-arid climate with typically low levels of humidity and cold winters with temperatures often dropping below freezing, and hot summers with temperatures rising above 30°C. During the fruiting season, temperatures generally range from 15°C to 25°C. A mixed climate for disease development exists during the fruiting season, when temperatures range from 15°C to 25°C. Low humidity and sporadic rainfall of 300-400 mm annually provide a dry environment that might stress apple plants and affect disease dynamics. Local strong winds can spread fungus spores and destroy trees. Quetta has sandy loam to loamy soil with somewhat alkaline pH levels, which affects plant health and nutrient availability. Sloped locations have more runoff and lower-lying places may store moisture (Pakistan Meteorological Department, 2023).

Disease Assessment and Sample Collection

The sampling was done across the 10 selected locations known for apple cultivation; Akhtarabad, Baleli, Chashma Achozai, Chiltan, Hanna Orak, Killi Gul Muhammad, Killi Hasni, Kuchlak, Qambrani, and Village Aid (Figure 1). At least three apple orchards were visited at each location for disease assessment by using the following disease incidence (DI) formula:

$$\mathrm{DI}(\%) = \frac{No.\,of\ Orchards\ with\ BRD\ Symptoms}{Total\ No.\,of\ Surveyed\ Orchards} \times 100$$

(Acheampong *et al.*, 2024):

A total of 300 symptomatic fruit samples showing characteristic symptoms of BRD were meticulously collected using a positive random sampling method (Sutton *et al.*, 2014).

Pathogen Isolation

Symptomatic samples were transported to the Fungal Plant Pathology Laboratory in the Department of Plant Pathology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi. To eliminate any contaminants and impurities, the infected samples were rinsed with tap water and dried on sterile filter paper. Fruit skin was then sliced into 5-10 mm^2 pieces including healthy margin using the sterilized surgical scalpel. These pieces were washed with 1% sodium hypochlorite solution for 45 seconds, rinsed with sterilized distilled water thrice and dried on sterile filter paper to remove remaining moisture. The direct plating technique was used for the isolation of *Colletotrichum* spp., placing the sterilized fruit skin pieces onto Petri plates containing Potato Dextrose Agar (PDA) media. The medium contained Streptomycin sulphate (100 mg L^{-1}) to prevent bacterial contamination (Hu *et al.*, 2023). Parafilm sealed Petri plates were incubated at 25±2°C temperature for 5-7 days. Purified cultures obtained by single spore technique (Du *et al.*, 2005). Details of sampling locations and recovered fungal isolates from symptomatic fruits is given in table 1.

Pathogenicity Tests

The pathogenic behavior of purified fungal isolates was assessed on healthy and mature apple fruits (*var.* Golden Delicious) in triplicate. A spore suspension was prepared by submerging a 14 days old pure culture in sterilized distilled water and dislodging the spores with a sterilized scalpel. Spore suspension, maintained at 1×10^6 ml^{-1} using a hemocytometer (Andrello *et al.*, 2024) was filtered through double-layered muslin cheesecloth to eliminate mycelial fragments. Healthy plant leaves and fruits were dipped in 1% sodium hypochlorite, followed by three washes with sterilized distilled water, and air-dried on sterilized blotter paper before spraying conidial suspension. The sterilized distilled water was sprayed on fruits as a control (Zhou *et al.*, 2023).

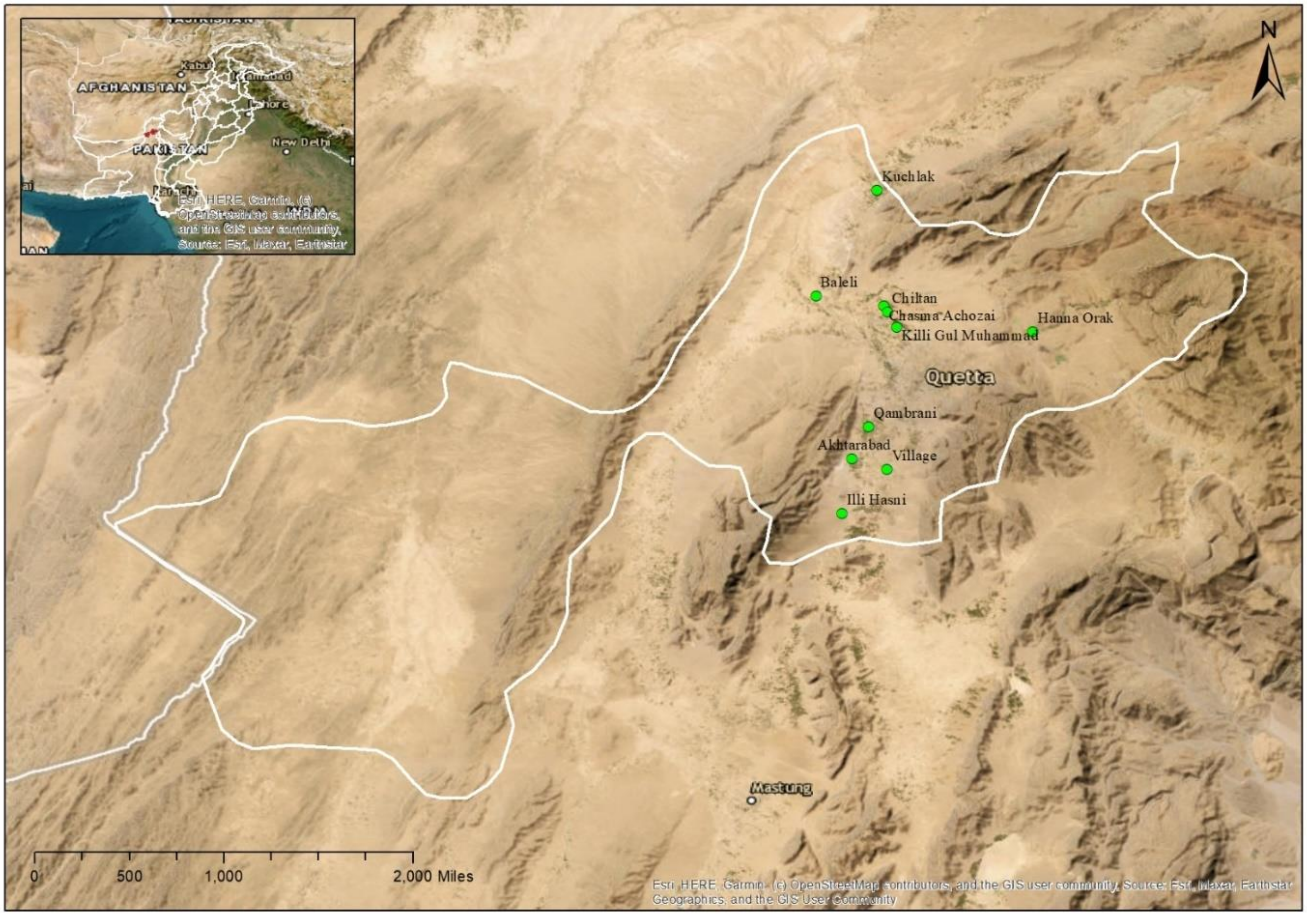

Figure 1. Geographical map of district Quetta highlighting ten locations surveyed for bitter rot disease documentation and sample collection.

Table 1. Details of sampling and obtained isolates during the survey.

Location (Geo-referencing)	Month	Purified isolates	Total Isolates Obtained
Akhtarabad	August	4	12
30°07'29.7"N / 66°56'40.0"E	September	8	
Baleli	August	5	13
30°16'48.5"N / 66°54'34.9"E	September	8	
Chashma Achozai	August	6	13
30°16'15.7"N / 66°58'28.7"E	September	7	
Chiltan	August	3	10
30°15'55.4"N / 66°58'41.2"E	September	7	
Hanna Orak	August	6	14
30°14'46.5"N / 67°07'03.4"E	September	8	
Killi Gul Muhammad	August	6	13
30°15'01.0"N / 66°59'12.9"E	September	7	
Killi Hasni	August	5	16
30°01'01.1"N / 66°57'46.9"E	September	11	
Kuchlak	August	1	10
30°22'52.4"N / 66°58'04.9"E	September	9	
Qambrani	August	2	13
30°09'19.2"N / 66°57'36.5"E	September	11	
Village Aid	August	5	16
30°06'52.9" / 66°58'38.7"E	September	11	

The inoculated leaves and fruits were kept in an incubator, maintaining 25±2 °C and >90% relative humidity (RH). The symptoms were assessed 7 days after inoculation (DAI) using a modified scale of 0–3: 0 (avirulent), no infection; 1 (slightly virulent), 1-5% of the fruit surface with a lesion; 2 (moderately virulent), 6-25% of the fruit surface with a lesion; and 3 (highly virulent), >25% of the fruit surface with a lesion appearance (Riolo *et al.*, 2023).

Morphological Characterization

Fungal colonies maintained on PDA media were subjected to both cultural and morphological characterization. Visual analysis was done to assess colony features, including texture, margin, and both front- and reverse-plate colors. Microscopic examination focused on spore characteristics such as size, color, shape, septation, appressoria, and setae. For each isolate, twenty measurements were recorded and statistically analyzed (mean and standard error) using Statistix software. The results were then compared with data from previously published literature (Weir *et al.*, 2012; Damm *et al.*, 2009; Sharma *et al.*, 2015; Khodadadi *et al.*, 2020).

Molecular Characterization

The nucleotide sequence analysis of nineteen highly virulent isolates was conducted at two laboratories: (1) the Fungal Plant Pathology Laboratory, Department of Plant Pathology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan, and (2) the Dr. Gleason Laboratory, Department of Plant Pathology and Microbiology, Iowa State University, Ames, USA.

DNA was extracted using the PrepMan® Ultra Reagent Kit following the manufacturer's protocol. PCR amplification was performed with primers targeting the internal transcribed spacer (ITS), beta-tubulin (TUB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes for pathogen and species confirmation, as described in the literature (White *et al.*, 1990; Martin *et al.*, 2021). Details of the PCR conditions are provided in Table 2.

A 1% agarose gel was prepared for electrophoresis of PCR products, with Gel Green used for nucleic acid staining. A standard 1 kb DNA ladder was employed for size comparison. Electrophoresis was conducted at 100 volts for 30-45 minutes, and DNA bands were visualized using the Gel Doc XR+ system (Bio-Rad, USA), which provides rapid, precise, and automated imaging.

Following electrophoresis, PCR products were purified and quantified using the QIAquick kit (according to the

manufacturer's protocol). The purified DNA was then submitted to the DNA facility at the Molecular Biology Building, Iowa State University, Ames, USA, for sequencing.

Received DNA sequences were aligned using BioEdit and compared with the GenBank database via BLAST for accession number assignment. Finally, homology was assessed using MEGA software, and a phylogenetic tree was constructed to determine the relationships among the isolates.

Table 2. Details of primers used in molecular analysis of *Colletotrichum* spp.

Locus	Primer	Primer Sequence	PCR Condition	Reference
Internal Transcribed Spacer region	ITS1 ITS4	5 TCCGTAGGTGAACCTGCGG 3 5 TCCTCCGCTTATTGATATGC 3	2 Min @ 95°C 35 cycles 60 Sec @ 94°C 60 Sec @ 56°C 60 Se @ 72°C 10 Min @ 72°C	(Martin *et al.*, 2021; White *et al.*, 1990)
Beta (β)-tubulin	TUB-F TUB-R	5 GGTAACCAAATGGTGCTGCTTTC 3 5 ACCCTCAGTGTAGTGACCCTTGGC 3	3 Min @ 94°C 34 cycles 60 Sec @ 94°C, 60 Sec @ 57°C, 60 Se @ 72°C 5 Min @ 72°C	(Martin *et al.*, 2021)
Glyceraldehyde-3-phosphate dehydrogenase	GPD-F GPD-R	5 ACCCCACTCGTTGTCGTACCA 3 5 ATTGACATCGTCGCTGTCAACGA 3	5 Min @ 94°C 35 cycles 30 Sec @ 94°C 30 Sec @ 55°C 90 Sec @ 72°C 10 Min @ 72°C	(Martin *et al.*, 2021)

RESULTS

Surveillance for Disease Assessment

The analysis of bitter rot incidence across different locations revealed significant variability. Baleli exhibited the highest mean incidence, with an average of 47.22%, showing 51.11% in Season 1 and 43.33% in Season 2. Killi Hasni followed with a mean incidence of 44.44%, recording 48.89% in Season 1 and 40.00% in Season 2. Chashma Achozai and Hanna Orak both had mean incidences of 39.44%, with Chashma Achozai showing 42.22% in Season 1 and 36.67% in Season 2, and Hanna Orak presenting 43.33% in Season 1 and 35.56% in Season 2. Kuchlak also had a mean incidence of 39.44%, with values of 43.33% in Season 1 and 35.56% in Season 2. Killi Gul Muhammad had a mean incidence of 37.22%, with 40.00% in Season 1 and 34.44% in Season 2. In contrast, Akhtarabad had a lower mean incidence of 33.33%, with 37.78% in Season 1 and 28.89% in Season 2. Qambrani showed a mean incidence of 34.44%, with 36.67% in Season 1 and 32.22% in Season 2. Chiltan had a mean incidence of 30.55%, with 32.22% in Season 1 and 28.89% in Season 2. Village Aid recorded the lowest mean incidence at 13.33%, with 16.67% in Season 1 and 10.00% in Season 2. These findings highlight the substantial variation in disease prevalence among different locations and seasons, underscoring the need for targeted management strategies.

Pathogenicity

A total of 130 isolates of *Colletotrichum* spp. were subjected to a pathogenicity tests, exhibiting symptoms of bitter rot disease in apples that were consistent with those observed in apple orchards during the survey. The pathogenicity test identified 40 avirulent, 31 virulent, 23 moderately virulent, and 36 highly virulent isolates. These isolates were further subjected to re-isolation to verify Koch's postulates (Figure 3).

Table 3. Percent mean incidence of bitter rot (caused by *Colletotrichum* spp.) at ten locations of Quetta district

Location	Season 1	Season 2	Mean
Akhtarabad	37.78 (±8.39)	28.89 (±13.47)	33.33
Baleli	51.11 (±5.09)	43.33 (±8.82)	47.22
Chashma Achozai	42.22 (±5.09)	36.67 (±6.67)	39.44
Chiltan	32.22 (±27.95)	28.89 (±25.02)	30.55
Hanna Orak	43.33 (±8.82)	35.56 (±6.94)	39.44
Killi Gul Muhammad	40.00 (±5.77)	34.44 (±5.09)	37.22
Killi Hasni	48.89 (±13.47)	40.00 (±6.67)	44.44
Kuchlak	43.33 (±3.33)	35.56 (±5.09)	39.44
Qambrani	36.67 (±3.33)	32.22 (±1.92)	34.44
Village Aid	16.67 (±8.82)	10.00 (±8.82)	13.33

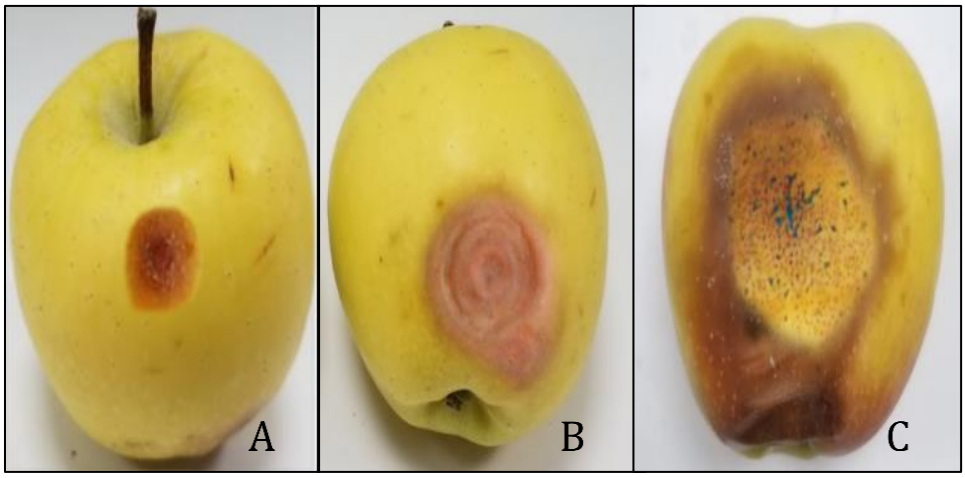

Figure 3**.** Pathogenicity outcomes of *Colletotrichum* spp. illustrating the symptoms and categorized into: slightly (A), moderately (B), and highly (C) virulent.

Cultural and Morphological Characterization

Fungal isolates grown on PDA media were classified into three categories based on their cultural and physical characteristics. Group A contained a total of 47 isolates. These isolates exhibited a pale white growth with an orangish spherical border, off-white to yellowish seeping in the core, and an orangish reverse. Group B had 41 isolates that had a grayish-white aerial mycelium with three to four hazy rings of pale yellowish hue in the centre, a whitish circular perimeter, and a pale yellowish reverse. Group C had 42 isolates, each with a brownish reverse, pale brown cottony aerial mycelium, greyish spherical borders, and two to three middle brown hazy rings. The conidia sizes varied greatly across the groups. Group-A isolates had conidial lengths of 13.5-18.7 µm and widths of 3.4-6.3 µm. Group-B isolates had lengths of 11.2 to 15.8 µm and widths of 3.7 to 5.6 µm. Group-C isolates had lengths of 11.2-14.7 µm and widths of 3.5-5.5 µm. None of the conidia had conidial septation; they were uniformly hyaline, tubular with tapering ends, and cylindrical. Group-A appressoria ranged in size from 4.5 to 10.5 µm in length and 4.7 to 7.5 µm in breadth. These appressoria were brown and ranged in shape from obovate to ellipsoid. The appressoria in Group-B ranged in length from 5.4 to 9.3 µm and in width from 3.5 to 7.5 µm. These appressoria had an oval to obovate shape and were medium brown in hue. Group-C appressoria measured 5.5–10.9 µm in length and 4.3–6.8 µm in breadth. These appressoria had an elliptic shape and were dark brown in hue. Importantly, Groups A and C lacked setae, whereas Group B isolates formed saw-like setae with one septation and a medium brown hue (Table 4 and Figure 4).

Table 4. Morphological characterization of the recovered fungal isolates.

Group	Colony Color	Conidia	Appressoria	Setae	Isolates
A	Pale white growth with orangish spherical border, Off white to yellowish seeping in core, Orangish reverse	13.5 – 18.7 µm (Length) 3.4 – 6.3 (Width) No septation Hyaline Tubular with tapering ends and cylindrical	4.5 to 10.5 µm (Length) 4.7 to 7.5 µm (Breadth) brown Obovate to ellipsoid in shape	Lacking	47
B	grayish-white aerial mycelium with three to four hazy rings of pale yellowish hue in the centre, a whitish circular perimeter, and a pale yellowish reverse	11.2 – 15.8 µm (Length) 3.7 – 5.6 (Width) No septation Hyaline Tubular with tapering ends and cylindrical	5.4 to 9.3 µm (Length) 3.5 to 7.5 µm (Breadth) Medium brown in hue Oval to obovate in shape	Saw-like in shape with one septation Medium brown in hue	41
C	brownish reverse, pale brown cottony aerial mycelium, greyish spherical borders, and two to three middle brown hazy rings	11.2 – 14.7 µm (Length) 3.5 – 5.5 (Width) No septation Hyaline Tubular with tapering ends and cylindrical	5.5 to 10.9 µm (Length) 4.3 to 6.8 µm (Breadth) Dark brown in hue Elliptic in shape	Lacking	42

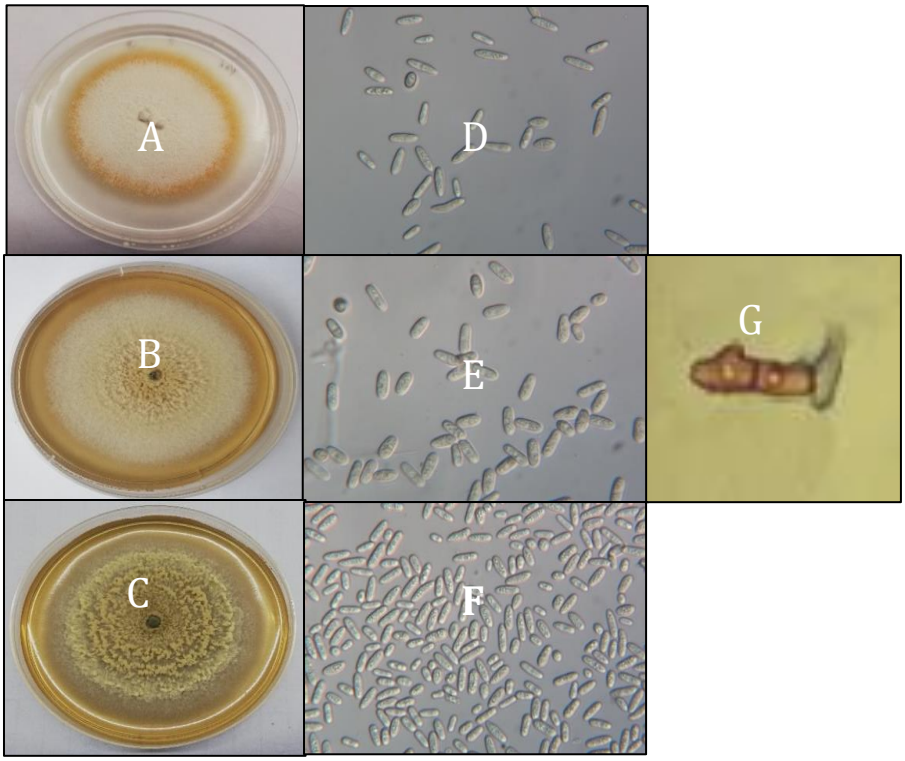

Figure 4. Pure colony of *Colletotrichum acutatum* (A), *Colletotrichum glodosporioides* (B), *Colletotrichum siamense* (C), with conidia of *C. acutatum* (D), *C. gloeosporioides* (E), *C. siamense* (F), and setae of *C. gloeosporioides* (G).

Molecular Characterization

Five highly pathogenic type isolates from Group A (A3JF316, CA1JF316, K1JF116, KH3JL517, and Q2JF517), six from Group B (B1JF216, B3JF516, CA1JF116, C2JF216, CA2JF317, and C1JF317), and eight from Group C (H1JF116, H2JF216, KH1JF416, KH2JF116, KH3JF516, A3JF217, H1JF117, and H2JF417) were selected for multi-locus sequence analysis using ITS, TUB, and GAPDH primers in a PCR assay. The resulting PCR products were run on a 1% agarose gel, visualized with a gel-documentation system, quantified, and submitted to the DNA Facility Center at Iowa State University (USA) for sequencing. The sequences obtained were compared with those in the GenBank NCBI database and exhibited 99–100% homology with previously submitted sequences of *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, and *Colletotrichum siamense*, respectively. Phylogenetic trees were constructed using maximum parsimony analysis on MEGA for the genes ITS, TUB, and GAPDH. The *Fusarium fujikuroi* reference sequence (MT553349) was obtained from the GenBank database. For *Colletotrichum acutatum*, the tree was divided into three clades (I=ITS, II=TUB, III=GAPDH). The first clade showed a 77% bootstrap value with type strains BBA-71331 (Germany) and SS-11 (Latvia). The second clade matched 97% with type strains STE-U-164 (South Africa) and BRIP:54787 (Australia). The third clade exhibited 34% similarity with type strains BRIP-62860 (Australia) and PJ57 (New Zealand) (Figure 5). Similarly, for *Colletotrichum gloeosporioides*, three clades were observed (I=ITS, II=TUB, III=GAPDH). The first clade showed an 80% bootstrap value with type strains CBS-L43 (China) and HC292 (USA). The second clade matched 94% with type strain LJWG01 (China) and 86% with JXYX-34 (China). The third clade showed 37% similarity with type strains ZZ-Y15 (China) and ZJF-2 (China) (Figure 6). For *Colletotrichum siamense*, the first clade with ITS exhibited a 75% bootstrap value with type strains SB73 (Malaysia) and CZ7 (China). The second clade (TUB) matched 90% with type strain ICKa38 (South Korea) and 54% with DF-43 (China). The third clade (GAPDH) displayed 34% similarity with type strains PC32 (China) and KY8 (USA) (Figure 7). The details of the obtained sequence numbers for study isolates are provided in Table 5.

Table 5. Details of accession no. obtained from GenBank, NCBI

Sr.	Pathogen	Isolates	Primer	Accession No.	Primer	Accession No.	Primer	Accession No.
1	*C. Acutatum*	A_3JF_3 16	GAPDH	ON861880	TUB	ON862015	ITS	OM765045
2	*C. acutatum*	CA_1JF_3 16	GAPDH	ON861882	TUB	ON862017	ITS	OM765047
3	*C. acutatum*	K_1JF_1 16	GAPDH	ON861885	TUB	ON862020	ITS	OM765050
4	*C. acutatum*	KH_3JF_5 17	GAPDH	ON861895	TUB	ON862030	ITS	OM765060
5	*C. acutatum*	Q_2JF_5 17	GAPDH	ON861898	TUB	ON862033	ITS	OM765063
6	*C. gloeosporioides*	B_1JF_2 16	GAPDH	OL989893	TUB	ON861997	ITS	OL875154
7	*C. gloeosporioides*	B_3JF_5 16	GAPDH	OL989895	TUB	ON861999	ITS	OL875156
8	*C. gloeosporioides*	CA_1JF_1 16	GAPDH	OL989896	TUB	ON862000	ITS	OL875157
9	*C. gloeosporioides*	C_2JF_2 16	GAPDH	OL989900	TUB	ON862004	ITS	OL875161
10	*C. gloeosporioides*	CA_2JF_3 17			TUB	ON862011	ITS	OL875168
11	*C. gloeosporioides*	C_1JF_3 17			TUB	ON862013	ITS	OL875170
12	*C. siamense*	H_1JF_1 16	GAPDH	ON886012	TUB	ON861920	ITS	OL875246
13	*C. Siamense*	H_2JF_2 16	GAPDH	ON886013	TUB	ON861921	ITS	OL875247
14	*C. siamense*	KH_1JF_4 16	GAPDH	ON886016	TUB	ON861924	ITS	OL875250
15	*C. siamense*	KH_2JF_1 16	GAPDH	ON886017	TUB	ON861925	ITS	OL875251
16	*C. siamense*	KH_3JF_5 16	GAPDH	ON886019	TUB	ON861927	ITS	OL875253
17	*C. siamense*	A_3JF_2 17	GAPDH	ON886021	TUB	ON861929	ITS	OL875255
18	*C. siamense*	H_1JF_1 17	GAPDH	ON886024	TUB	ON861932	ITS	OL875258
19	*C. siamense*	H_2JF_4 17	GAPDH	ON886025	TUB	ON861933	ITS	OL875259

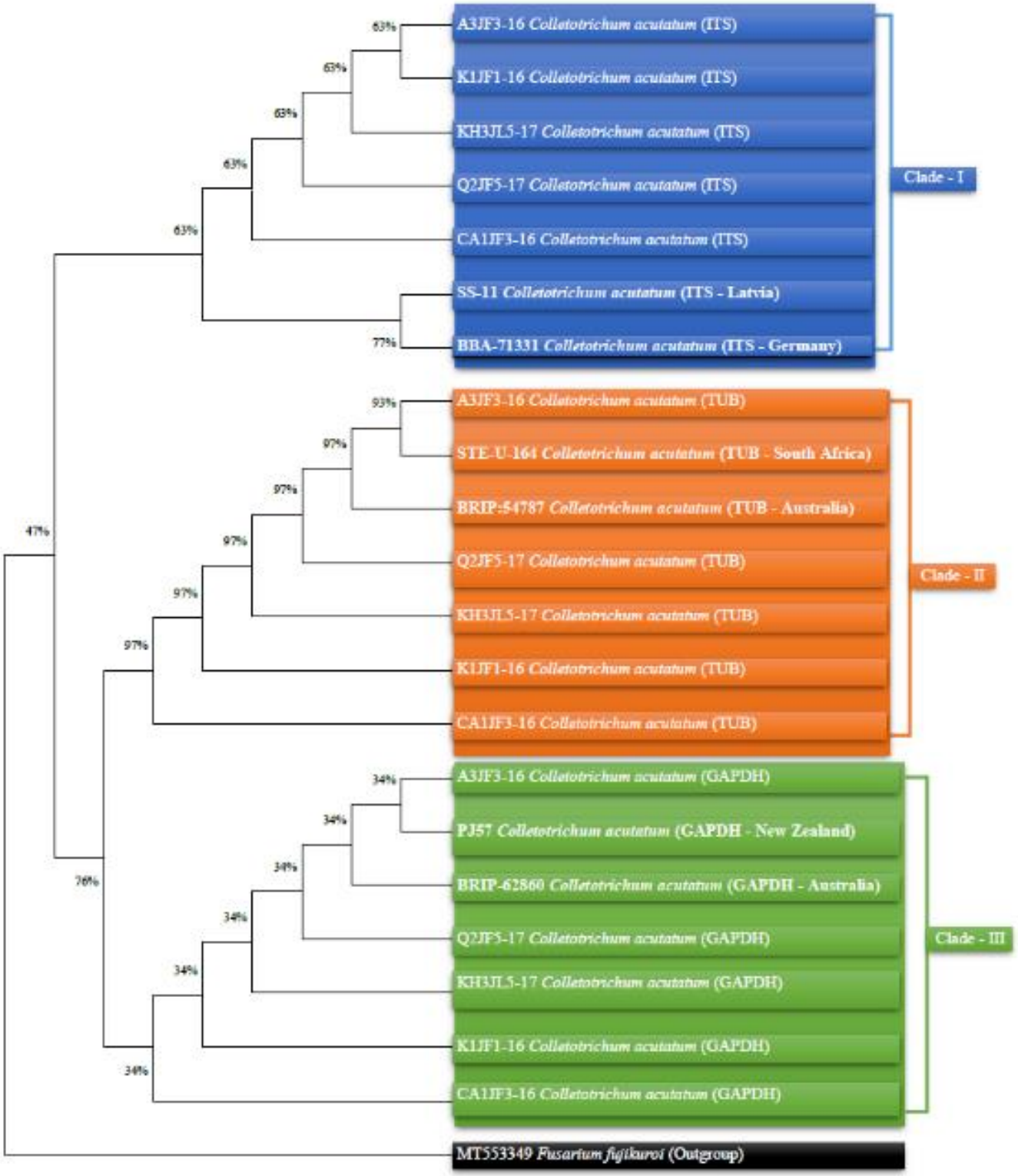

Figure 5. Phylogenetic relationship of the *Colletotrichum acutatum* with already published and reported species around the globe.

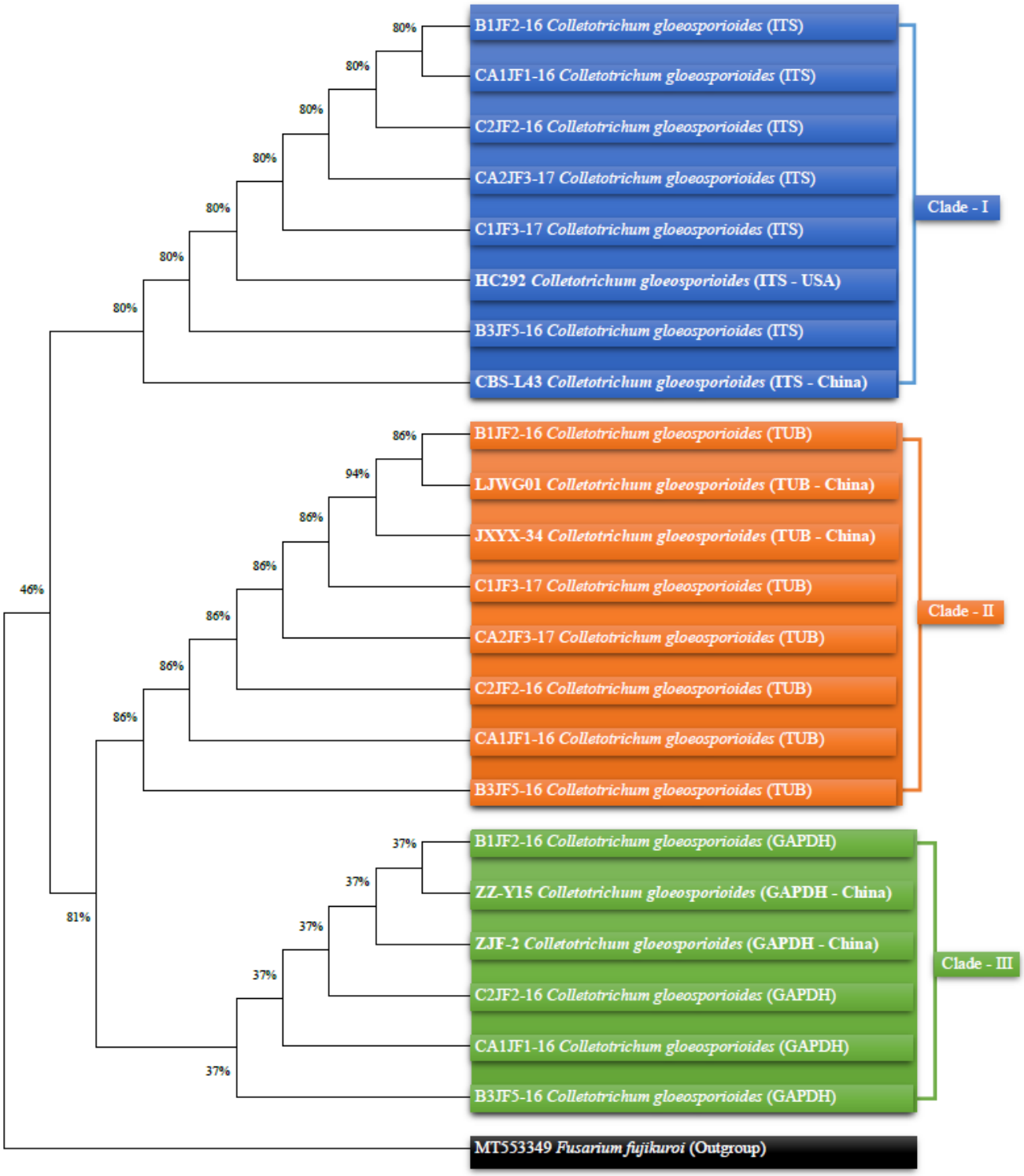

Figure 6. Phylogenetic relationship of the *Colletotrichum gloeosporioides* with already published and reported species around the globe

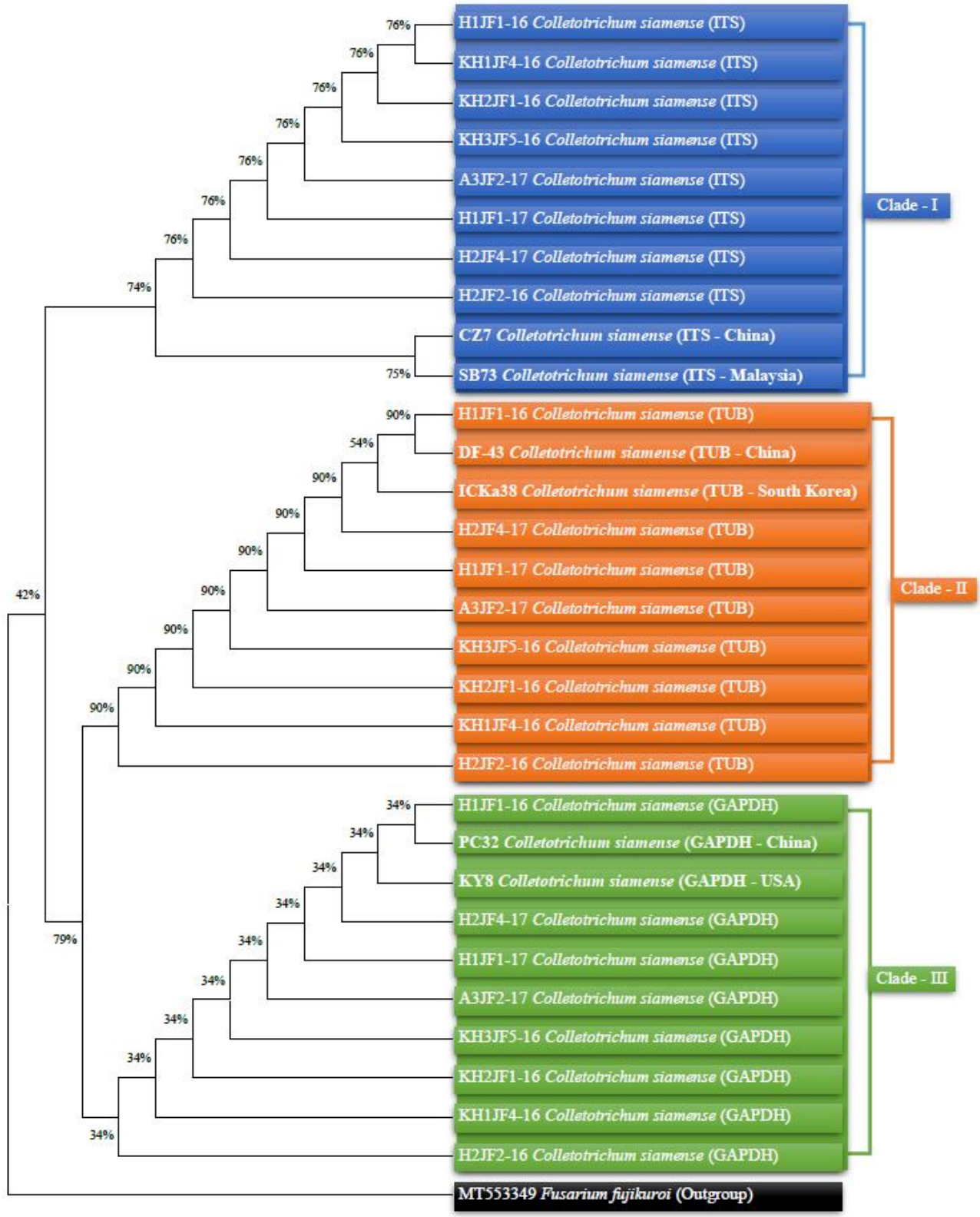

Figure 7. Phylogenetic relationship of the *Colletotrichum siamense* with already published and reported species around the globe

DISCUSSION

Bitter rot, a prevalent disease, affects nearly all apple cultivation regions globally, encompassing areas such as China (Chen *et al.*, 2022), the USA (Munir *et al.*, 2016), Brazil (Velho *et al.*, 2014), Italy (Carneiro and Baric, 2021), Belgium (Grammen *et al.*, 2019), and Croatia (Ivic *et al.*, 2013). This disease is causing substantial economic losses due to favorable environmental conditions. This study marks, to the best of our knowledge, the first comprehensive investigation into the role of *Colletotrichum* spp. in causing the bitter rot disease of apples in Pakistan. We conducted a survey at ten locations in Quetta to evaluate the disease assessment and collect samples for this study. We calculated the disease incidence at 39.22% in season 1 but found it at 32.56% in season 2. Unfavorable weather conditions, which were less conducive to the survival and progress of *Colletotrichum* spp. for disease development, may have contributed to a reduction in disease prevalence and incidence in 2017. According to González and Sutton (2004), the study revealed that prolonged warm and wet weather conditions are responsible for pathogen survival and the development of bitter rot in apples. Another study by Nekoduka *et al.* (2018) concluded that epidemiological factors (rainfall, humidity, and temperature) play a critical role in the development of bitter rot.

Our observations identified the symptoms of bitter rot in apple orchards, which are characterized by small, sunken lesions appearing on the fruit's outer surfaces. As these lesions progress, they remain sunken and circular in shape, potentially developing red halos. Over time, these lesions darken to brown, forming a distinctive bullseye pattern. Fernandez *et al.* (2022) have reported comparable symptoms in a low-chill apple cultivar grown in central Argentina. In this study, we brought 300 bitter rot disease (BRD)-infected samples to the Fungal Plant Pathology Laboratory for isolation, pathogenic behavior confirmation, and cultural and morphological characterization; however, we successfully purified only 130 isolates. Transportation damage contributed to the reduction in numbers, and the laboratory isolation process revealed contamination in some isolates. Pathogenicity tests revealed that 36 out of 130 isolates were highly virulent and developed similar symptoms of bitter rot on apples as observed during sample collection.

We classified 130 purified *Colletotrichum* spp. isolates into three groups (A, B, and C) in our study, taking into account variations in their cultural (Colony color) and microscopical (Conidia, appressoria, and setea) characteristics. It is imperative to consider the historical reliance on morphological and cultural characteristics for identifying *Colletotrichum*. Various studies, including those by Arx (1957), Sutton *et al.* (2014), Bailey and Jeger (1992), have highlighted the significance of these traits in classical identification systems. Nevertheless, Sutton *et al.* (1992) pointed out the insufficiency of morphology alone for precise identification. Crouch *et al.* (2009) made a pivotal observation about the limited taxonomic formativeness and practical utility of features such as conidial size, shape, and appressoria for species identification and confirmation. Environmental factors, media choice, incubation conditions, and temperature can induce changes in these features (Cannon *et al.*, 2000). Cai *et al.* (2009) acknowledged the challenge of comparing morphological and cultural characteristics due to variations and inconsistencies in methodology. For meaningful comparisons among isolates or species, it is essential to adopt a standardized fungal cultivation protocol. Digging deeper, a comprehensive awareness of the constraints within the traditional identification system is crucial to enhancing the efficacy of disease management. Some findings indicate the need to complement morphological characters with other traits for a better species relationship (Crouch *et al.*, 2009; Prihastuti *et al.*, 2009). This prompts the molecular study of the pathogen. Considering all the aforementioned weaknesses, researchers have recognized nucleic acid sequence analysis as a more reliable approach for the classification and specie identification of *Colletotrichum* spp. (Sutton *et al.*, 1992; Cannon *et al.*, 2000; Crouch *et al.*, 2009; Damm *et al.*, 2009; Prihastuti *et al.*, 2009).

Therefore, we employ a multi-gene phylogeny approach to systematically characterize relationships and identify species within *Colletotrichum*. Three genes—internal transcribed spacer (ITS), beta tubulin (TUB), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)—were used to confirm that the species was *Colletotrichum*. These gene regions aided in illustrating the phylogenetic relationship among three *Colletotrichum* spp., specifically Group (A) *C. acutatum*, Group (B) *C. gloeosporioides*, and Group (C) *C. siamense*, which cause bitter rot in apples across various countries. They also provided an overall characterization of the *Colletotrichum* spp. responsible

for this disease. Hence, the present results align with findings from a prior study (Munir *et al.*, 2016). Our research aligns with the previous study by Oo *et al.* (2018), which used two gene regions, ITS and GAPDH, to confirm the presence of *Colletotrichum* spp., specifically *C. acutatum*, *C. gloeosporioides*, and *C. siamense*, which cause the bitter rot disease of apples. Similarly, previous studies emphasize the utility of molecular studies for species-level identification, especially when morphological characterization poses challenges (Lee *et al.*, 2007). Based on our findings, we report for the first time the occurrence of *Colletotrichum* spp. causing bitter rot disease of apples in Pakistan, highlighting the significance of molecular investigations of *Colletotrichum* spp. at the species level.

CONCLUSION

Notably, no systematic study on bitter rot disease of apple had been conducted in Quetta prior to this research. Thus, this study represents the first comprehensive assessment and characterization of *Colletotrichum* species responsible for bitter rot in Quetta, Balochistan. The findings provide detailed insights into the *Colletotrichum* species prevalent in the region and their role in causing bitter rot. Future research can now focus on developing management strategies based on this documented characterization, rather than re-characterizing these fungal pathogens.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally to the research and compiling the data as well as editing the manuscript.

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